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(57) Abstract

The present invention relates to methods for diagnosing vaginal disorders associated with various microorganisms that may be used in a medical practitioner's private office or in a more structured clinical environment, such as a hospital, a commercial clinical microbiology laboratory or the like. In addition, methods for simultaneously selectively detecting gram positive bacteria and at least one other microbial organism which is not a gram negative bacterial species.

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METHODS AND PHARMACEUTICAL KITS USEFUL FOR DETECTING MICROORGANISMS ASSOCIATED WITH VAGINAL INFECTIONS

Technical Field

The present invention relates to methods for diagnosing vaginal disorders associated with various microorganisms that may be used in a medical practitioner's private office or in a more structured clinical environment, such as a hospital, a commercial clinical microbiology laboratory or the like. In addition, methods for simultaneously selectively detecting gram positive bacteria and at least one other microbial organism which is not a gram negative bacterial species.

Background of the Invention

Bacterial vaginosis (BV) is characterized by an alteration of the microbiological flora of a woman's vaginal area. This alteration involves a reduction in the normally present Lactobacilli cell number and an overgrowth of anaerobes and other microorganisms, including Gardnerella vaginalis (Gv). Other microorganisms also cause vaginal disorders as well.

BV is one common cause of vaginal complaints. Other microorganisms commonly associated with such symptoms are Candida spp. and Trichomonas vaginalis. The

Summary of the Invention

The present invention provides methods for detecting the presence of various microorganisms that are found in vaginal samples. These methods are fast, accurate, and do not require an individual skilled in identifying clue cells, evaluating wet mounts or the like to assess the results.

Methods for simultaneously selectively detecting gram positive bacteria and at least one other microbial organism which is not a gram positive bacterial species (non-gram positive microbe) in a single complex biological sample are disclosed. They comprise:

(a) lysing the cells of the gram positive bacteria and the non-gram positive microbe to release nucleic acid from said organisms by combining a lysis solution with the single sample and contacting the released nucleic acid with an oligonucleotide probe specific for the gram positive bacteria and an oligonucleotide probe specific for the non-gram positive microbe to form a gram positive bacteria-probe hybridization complex and a non-gram positive microbe-probe complex, respectively; then

(b) detecting both of the hybridization complexes.

The gram positive bacteria may be a Group B Streptococci and the non-gram positive microbe may be selected from the group consisting of yeasts, protozoa, mycoplasmas, and gram negative bacteria. In a preferred embodiment, the non-gram positive microbe is Gardnerella vaginalis.

In one embodiment of such methods, the oligonucleotide probe specific for the gram positive bacteria coats a first capture bead and the

oligonucleotide probe specific for the non-gram positive microbe coats a second capture bead, both beads being attached to a dipstick comprising a nonporous solid support.

Further, the gram positive-probe hybridization complex may be detected by a first signal oligonucleotide and the non-gram positive microbe-probe hybridization complex may be detected by a second signal oligonucleotide.

Further disclosed is a method for releasing intact nucleic acid from gram positive bacteria comprising:

combining a complex biological sample containing gram positive bacteria with a lysis solution of about pH 7.0 to about 8.0 containing a low ionic strength buffer and a detergent; then heating the combined solution to above 65°C for more than five minutes to release intact nucleic acid from gram positive bacteria in the absence of mechanical force. Intact nucleic acid from gram negative bacteria may also be released from gram negative bacteria within the biological sample.

The kits of the present invention can also contain dipsticks capable of specifically capturing nucleic acid from one or more of Group B Streptococci, Prevotella bivia, Ureaplasma urealyticum, Mobiluncus spp., Bacteroides spp., Mycoplasma spp., Neisseria gonorrhea and Chlamydia spp. in addition to or in lieu of Gardnerella vaginalis, Candida spp. or Trichomonas vaginalis.

Detailed Description of the Invention

The present invention is directed to methods and kits useful in the diagnosis of microorganisms in vaginal fluid samples. The invention provides means for lysing gram positive bacteria so that nucleic acid is released intact and is available for hybridization assays or other diagnostic or clinical applications. Lysis of such bacteria has heretofore been problematic, requiring mechanical means and equipment such as a French press. Further, the gram positive bacterial nucleic acid can be released along with that of other non-gram positive bacterial microbial organisms in the same sample, all such nucleic acid being available for hybridization assays.

As an example of determining whether an abnormal amount of microorganism exists in a biological sample, such as vaginal fluid, a G. vaginalis cell number greater than or equal to a critical G. vaginalis cell number, indicates that the patient is BV- positive.

In almost every instance, the vaginal pH in women with BV is higher than pH 4.5, whereas the vaginal pH of women who are clinically negative for BV is almost always less than 4.5. However, pH alone is not diagnostic for BV, because other conditions can lead to elevated vaginal pH. While women with BV have elevated levels of G. vaginalis in their vaginal tract, enumeration of G. vaginalis, taken alone, is not a good indicator of BV, because BV-negative women frequently have elevated levels of G. vaginalis in their vaginas. The present invention permits rapid measurement of pH and G. vaginalis levels in a patient sample, as an indication of Bacterial Vaginosis.

The claimed methods and kits involve determining whether the G. vaginalis cell number in a patient sample is greater than or equal to a critical G. vaginalis cell

number. The critical G. vaginalis cell number is a predetermined cell number that is associated with BV. The critical G. vaginalis cell number may vary with the sample collection method employed, and is defined herein to be either cell number or cell concentration. To initially identify the critical G. vaginalis cell number corresponding to a particular sampling method and assay procedure, the sampling method will be used to obtain specimens from a sufficient number of women that have been clinically diagnosed as BV-positive or BV-negative to establish the critical G. vaginalis cell number. A range of G. vaginalis cell numbers from the BV-positive women will be determined, and the critical G. vaginalis cell number will represent the bottom of this range. This critical number is tied to the selected sampling method. Determination of the critical G. vaginalis cell number will be dependent upon the sample collection procedure, such as vaginal wash, vaginal swab, or other sample obtaining means, as well as the assay used to measure the number of G. vaginalis cells present in that sample within a period of 6 hours or less. The G. vaginalis cell number may be measured by nucleic acid hybridization techniques, by techniques involving antibody-antigen interactions, or by any other method whose results can be correlated with a G. vaginalis cell number or G. vaginalis cell concentration. While the critical G. vaginalis cell number may be dependent on the methods chosen for sample collection and for G. vaginalis enumeration, the critical G. vaginalis cell number will be consistent over a 10-fold range for any selected protocol.

Using the selected sampling method and assay procedure, the G. vaginalis cell number of the sample will

standards derived from a known number of purified, cultured G. vaginalis. Serial dilutions of quantitated, cultured G. vaginalis are subjected to the same assay procedures as the patient samples. The signal intensity of each patient sample is compared to signal intensities of the diluted standards, and the patient sample can be correlated with an amount of G. vaginalis equivalent to that in the matching standard dilution.

Alternatively, a specific molecule that serves as the target of the G. vaginalis assay can be purified from a known number of cultured G. vaginalis and used as a standard for comparison to the patient sample results. For example, if 16S rRNA is the target of the assay, purified 16S rRNA could serve as the standard, with a given amount of 16S rRNA corresponding to a known number of G. vaginalis cells. In this instance, the patient sample results could be expressed in units equivalent to molecules of 16S rRNA; BV-positive and BV-negative women are characterized according to how many 16S rRNA equivalents are present in the sample. This approach could be used for any other purified molecule obtained from a known number of G. vaginalis cells. If desired, a separate experiment could determine the number of 16S rRNA molecules per G. vaginalis cell, and thereafter the 16S rRNA standard could be used, but the results could be expressed per G. vaginalis cell number. Since the number of target molecules (such as 16S rRNA) per cell can vary during different stages in the G. vaginalis cell growth cycle, the number of correlate molecules per cell should be established using a known number of G. vaginalis cells that are in the mid-log phase of growth. For consistency, this numerical equivalency will be determined only once, and thereafter the purified target molecule, such as 16S

rRNA, can be used as a standard, with conversion to numbers of G. vaginalis cells according to the established numerical equivalency.

Once the selected method has been used to determine the number of G. vaginalis cells per sample from the BV-positive and the BV-negative groups, the critical G. vaginalis cell number may be determined (i.e., the minimum number of G. vaginalis cells associated with BV-positive women). While some of the BV-negative women may have G. vaginalis cell numbers greater than the critical level, these women will nearly always be found to have a vaginal pH < 4.5, and thus will be diagnosed as BV-negative when the diagnostic kit instructions are followed.

Within the present invention, a critical cell number may be determined for an organism other than G. vaginalis in a manner similar to that set forth above for determining critical G. vaginalis cell number. For instance, the critical cell number for women presenting with Candidiasis may be determined relative to indigenous Candida spp. levels found in the vagina. For organisms not normally found in vaginal fluid, no critical cell number exists. The presence of such cells in any number indicates an abnormality. For example, the lower detection limit of the kit for T. vaginalis under ideal conditions is about 5×10^3 cells. This cell number or one as close to it as possible will be used to indicate positive results of a T. vaginalis probe.

The concentrations of the reaction components of a solid phase assay kit of the present invention will be adjusted to ensure that a positive signal will be obtained only if the amount of G. vaginalis present is equal to or

for G. vaginalis is about 2 x 10^5 cells. The critical G. vaginalis cell number will generally range from about 5 x 10^6 to about 5 x 10^9 cells per ml of vaginal fluid, with about 8 x 10^6 to about 10^9 cell/ml of vaginal fluid preferred, and about 2 x 10^7 cells/ml of vaginal fluid particularly preferred. For the purposes of this description, "about 2 x 10^7 cells/ml of vaginal fluid" means a G. vaginalis cell level within the range from about 5 x 10^6 cells/ml to about 5 x 10^9 cells/ml.

Vaginal fluid samples that may be tested in accordance with the present invention may be obtained in any conventional manner. Exemplary sample obtaining methodologies involve the use of a vaginal swab, vaginal wash techniques or the like.

The pH determining step of the methods of the invention may be accomplished by conventional techniques, such as contacting a sample, such as a vaginal swab or speculum, with pH paper or another pH indicating substrate and observing an alteration in the color of the paper or substrate that is indicative of relevant sample pH. The pH indicator may be included within a diagnostic kit of the present invention as a separate structural unit or as a portion of the structure of a diagnostic indicator card or dipstick. Alternatively, the pH indicator might be provided by the clinician. In addition, pH may be measured with electrodes or with commercially available pH indicators; alternatively, known pH indicators can be immobilized on a solid support.

Similarly, the G. vaginalis cell level may be determined by any method yielding an accurate measurement thereof that may be performed in 6 hours or less. Consequently, probes, including oligonucleotide sequences that are complementary to G. vaginalis DNA or RNA, antibodies selective for G. vaginalis or the like, may be

used for this purpose. Direct hybridization assay techniques may be used in making the G. vaginalis cell level determination. Sandwich assay techniques employing oligonucleotide probes or antibodies are preferred.

In another embodiment of the present invention, several microorganisms associated with vaginal complaint are detected simultaneously and rapidly using one sample preparation. When patients present with vaginal complaints, microorganisms commonly associated with such symptomology include Candida spp. and Trichomonas vaginalis, as well as Gardnerella vaginalis. Therefore, a method to quickly, easily and simultaneously detect the presence of one or more of these three organisms in a single biological sample would be advantageous. In addition, simultaneous detection of Gardnerella vaginalis and Trichomonas vaginalis is also diagnostically useful.

The claimed invention describes lysis conditions suitable for releasing target nucleic acid from each of the microorganisms above plus mycoplasma, gram positive bacteria, other yeasts, and other protozoa (representing both prokaryotic and eukaryotic cells). The released nucleic acids, and preferably released ribosomal RNAs, are then simultaneously processed and detected in a sandwich assay.

The present invention also contemplates diagnostic kits for determining whether a patient is afflicted with BV, including a first indicator capable of indicating pH; and a second indicator capable of indicating a G. vaginalis cell number or level associated with the disease state. Indicators of the presence of other organisms, such as Candida albicans, Trichomonas vaginalis, Neisseria gonorrhoea, Chlamydia spp.,

Streptococci or the like, may also be included in the kits of the present invention. In this manner, the kits of the present invention may be employed to detect BV, vaginitis or cervicitis.

Specifically, the kit may contain a strip of pH indicating paper and a diagnostic indicator card, such as a dipstick. A cell disruption buffer/hybridization solution or a ligand incubation solution (optionally containing appropriate signal moieties) may also be included within the kits of the present invention to facilitate employment of sandwich assay techniques. Alternatively, the pH indicator may be structurally integrated with the G. vaginalis cell number indicator. The kits may be used in manual or semi-automatic testing procedures, and are preferably employable in sandwich assay techniques.

When simultaneously detecting the presence of more than one target nucleic acid, several parameters are considered. For instance, lysis solutions that effectively release nucleic acid from each target microorganism must be determined. Exemplary lysis solutions will include a buffer, a detergent, and may also include other buffers, detergents, enzymes, reducing agents, organic solvents, antibiotics, chaotropic agents, cations, chelates, preservatives or combinations thereof.

A low ionic strength buffer is a buffer having a total ion concentration of about 15 mM to about 150 mM. Buffers useful in accordance with the present invention include, but not limited to, the following: brucine tetrahydrate, 4-(2-hydroxyethyl)-1-piperazinepropane sulfonic acid ("EPPS"), tris(hydroxymethyl)aminomethane ("TRIS"), N-tris(hydroxymethyl)methylglycine ("TRICINE"), glycinamide, N,N-bis(2-hydroxyethyl)glycine ("BICINE"), N-tris(hydroxymethyl)methyl-2-aminopropane sulfonic acid

("TAPS"), N-glycyl-glycine, histidine, boric acid, Pyrophosphoric acid, ethanolamine, glycine, trimethylamine, cyclopentanetetra-1,2,3,4-carboxylic, carbonic (H_2CO_3 + CO^2), 3-cyclohexylamino-1-propanesulfonic acid ("CAPS"), "EDTA", methylamine, dimethylamine, ethylamine, triethylamlne, diethylamine, ascorbic acid and phosphoric acid.

Detergents useful in the lysis solution of the present invention include the following:

Anionic detergents including but not limited to, the following: cxaprylic acid, sodium salt, cholic acid, sodium salt, 1-decanesulfonic Acid, sodium salt, deoxycholic acid, sodium salt; glycocholic acid, sodium salt; glycodeoxycholic acid, sodium salt; lauryl sulfate, sodium salt ("SDS"); N-lauroylsarcosine, sodium salt; taurocholic acid, sodium salt; and taurodeoxycholic acid, sodium salt;

Cationic detergents including but not limited to the following: cetylpyridinium chloride, dodecyltrimethylammonium bromide, hexadecyltrimethylammonium bromide, tetradecyltrimethylammonium bromide;

Zwitterionic detergents including but not limited to, the following: CHAPS and CHAPSO; and

Non-ionic detergents including but not limited to, the following: n-decyl-β-D-glucopyranoside, digitonin, n-dodecyl β-D-glucopyranoside, n-dodecyl-β-D-maltoside, n-heptyl β-D-glucopyranoside, n-octyl-β-D-glucopyranoside, n-octyl α-D-glucopyranoside, nonidet P-40, n-nonyl-β-D-glucopyranoside and Triton X-100.

For the lysis of gram positive bacteria, it has been found that it is preferred to use a lysis solution in

combined, the combined solution is heated for over five minutes at at least 65°C. This enables the lysis and subsequent release of the nucleic acid. The nucleic acid is "intact" in that it exists in sufficient form to be available for hybridization assays, i.e. fragments of at least 18 nucleotides are present.

Preferred capture oligonucleotides are selected for both target specificity and duplex (or hybridization-complex) formation with target in a solid phase sandwich assay format. The latter characteristic is particularly important when the target is ribosomal RNA. Signal oligonucleotides may be selected from a range of specificities. That is, signal oligonucleotides may be specific for prokaryotic target nucleic acid or eukaryotic target nucleic acid, or may specifically hybridize with the genus and/or species of the target. The selection of capture oligonucleotides, signal oligonucleotides, lysis conditions, concentration of capture oligonucleotide on a bead, hybridization conditions, signal/detection systems and conditions, and combinations thereof may impact the sensitivity and selectivity of detection of a particular panel of microorganisms. However, one skilled in the art of nucleic acid sandwich hybridization assays can determine modifications or adjustments of these parameters that optimize simultaneous detection of a particular panel of target microorganisms.

In solid phase sandwich assays involving oligonucleotide probe technology (described generally by Dunn et al., Cell 12:23-36, 1977), the target nucleic acid is usually released from the target microorganism into a solution. The target nucleic acid solution is then transferred to or adjusted to solution conditions in which hybridization to a solid support can occur. Such an assay technique may also be employed on a portion of the

original patient sample that has been manipulated in some manner (i.e., purified, treated chemically or the like).

In a preferred embodiment, target nucleic acid is typically sequestered (captured) from the original patient sample by hybridization (i.e., pairing of complementary bases) with capture oligonucleotide probes that are covalently immobilized on the surface of a solid support. Alternatively, the target nucleic acid from the original patient sample may be amplified to form an amplicon which is captured and hybridized to the signal probe. The captured target nucleic acid is then hybridized to a signal oligonucleotide probe having a detectable label bound thereto, or having the capability of binding to a moiety having a detectable label bound thereto. The signal probe is specific for an alternative site on the target nucleic acid. Alternatively, signal hybridization can be performed simultaneously with capture hybridization by including the signal probe within, for example, the hybridization solution. This results in a "sandwich" of the capture oligonucleotide probe:target nucleic acid:signal oligonucleotide probe. The solid support is washed to remove unhybridized material, and the labeled nucleic acid is then measured in accordance with detectable characteristics of the label.

In sandwich assays involving antigen/antibody technology, antigen is either present in the original sample, extracted therefrom or released from organisms contained in the original sample by reagents that disrupt the cell wall and/or membrane. The antigen is sequestered (captured) from the test sample by interaction with antigen specific antibody that is covalently immobilized on the surface of a solid support. The captured target

capability of binding to a moiety having a detectable label bound thereto. The signal antibody is specific for an alternative site on the target antigen. Alternatively, signal antibody binding can be performed simultaneously with capture by including the signal antibody within, for example, the incubation solution. This results in a "sandwich" of the capture antibody:target antigen:signal antibody. The solid support is washed to remove unbound signal antibody, and the labeled antigen is then measured in accordance with detectable characteristics of the label.

In the sandwich assays described above, biotin/avidin or biotin/streptavidin technology may also be employed. Specifically, the biotin/avidin interaction may be exploited to couple a more generalized detection system to the oligonucleotide probe or antigen/antibody sandwich assays. For example, the signal probe of the sandwich assay may be covalently bound to biotin. This biotin-labeled signal probe can then be incubated with avidin or streptavidin, its complementary ligand, having a detectable label bound thereto. This results in the detection of a "sandwich" of the capture oligonucleotide probe:target nucleic acid:signal oligonucleotide probe/biotin: avidin/detectable label. In this embodiment, avidin/detectable label can be prepared in large scale and can bind to signal/biotin moieties in a variety of sandwich assays. Other ligand pairs are also useful in the solid phase assays of the present invention. Exemplary of such additional ligand pairs are lectin:sugar, hormone:hormone receptor and the like.

The preferred solid support for use in the diagnostic methods and kits of the present invention is a polymer-coated bead having activatable amine groups, as described in copending U.S. Patent Applications Serial

Nos. 444,872 and 522,442. Bead solid supports useful in accordance with the present invention possess certain advantages over known membrane or bead solid supports, to which capture nucleic acid sequences are noncovalently bound. The capture rate of target nucleic acid sequences is improved 5- to 25-fold. Virtually all of the capture nucleic acid sequence bound to the solid support is available for hybridization with a complementary sequence. Also, the quantity of immobilized capture nucleic acid can be increased approximately 20-fold on an apparent surface area basis. The preferred solid supports may be more easily manufactured than their prior art counterparts. In addition, such beads possess covalently immobilized capture nucleic acid sequences (oligonucleotides) that can withstand denaturation temperatures in excess of 90°C for 10 or more minutes. Also, a multisite dipstick employing a plurality of bead solid supports can be constructed, leading to miniaturization of the dipstick device and reduction in the volume of sample required for detection. These advantages contribute to increased sensitivity when a sandwich assay hybridization format is used to diagnose BV in accordance with the present invention.

Capture nucleic acid sequences can be directly immobilized on a microtiter plate or on loose beads which are placed in the wells of a microtiter plate. Covalent immobilization of an oligonucleotide on a solid support, preferably a bead, may be accomplished by the following procedure: treating a solid support with an activating agent, if necessary; reacting the treated solid support with an amine-containing polymer, whereby the polymer coats the solid support; activating an oligonucleotide with a monofunctional or multifunctional reagent

and the polymer-coated solid support. The unreacted amines on the support surface are then acylated to impart the proper surface charge to the solid support surface, thereby minimizing non-specific interaction with nucleic acid.

For the purposes of this description, the term "nucleic acid" includes single stranded DNA and RNA, and double stranded DNA, RNA and DNA-RNA hybrids.

For the purposes of this description, the term "lysis" refers to rendering nucleic acids or antigens of cells contained in the test sample available to the capture probe or ligand. The cells acted upon by a lysing agent may or may not be structurally altered in a manner detectable by visual inspection under a microscope. Exemplary lysing solutions useful in practicing the present invention include detergents (e.g., anionic, cationic and/or non-ionic), chaotropic agents, enzymes (e.g., proteases, sialidases, glycosidases, glucanases, chitinases, lyticase, lysozyme, lipases, hyaluronidase, and the like), organic solvents, reducing agents (e.g., dithiothreitol, β-mercaptoethanol or the like), chelating agents (e.g., EDTA, EGTA and the like), antibiotics or the like, used alone or in combination. One preferred embodiment of the present invention involves pretreatment with one or more enzymes, organic solvent, or reducing agent prior to addition of a chaotropic agent. Lysis may be conducted at ambient or at elevated temperature.

For the purposes of this description, the term "solid support" refers to any surface that is transferable from solution to solution and that has means for attaching a bead. For instance, a bead may be attached to a perforation or depression in the solid support.

For the purposes of this description, the term "bead" refers to a structure for conducting

oligonucleotide-based assays, and includes beads, microbeads, powder, membranes, microtiter wells, strings, fabric, plastic strips, films, or any surface onto which nucleic acid probes or antigen may be immobilized. Solid supports useful in the practice of the present invention may exhibit natural or intrinsic fluorescence. For instance, the term "bead" encompasses any type of solid or hollow sphere, ball, bearing, cylinder, or other similar configuration composed of plastic, ceramic, metal, nylon, or polymeric material onto which a nucleic acid or antigen can be covalently immobilized. As such, the term also includes nylon string. Preferably, spherical nylon beads are employed in the methods and kits of the present invention. A preferred diameter range for such beads is from about 0.01 inch to about 0.5 inch, more preferably from about 0.05 inch to about 0.1 inch, and most preferably about 0.06 inch (corresponding to commercially available 3/32 inch nylon beads). Additionally, it is preferred that the nylon beads are burnished or roughened before treating with an alkylating agent.

In a preferred embodiment of the present invention, a nylon bead (or beads or any composition or structure of nylon) is activated by treating the bead with an alkylating agent. Alkylating agents react with amides present in the nylon polymer to form reactive imidate esters. Preferred alkylating agents include, but are not limited to, dialkyl sulfates, alkyl triflates, alkyl-diphenyl sulfonium salts, alkyl perchlorates, and, more preferably, trialkyloxonium salts. Exemplary trialkyl-oxonium salts useful in the present invention include lower alkyl salts, such as trimethyloxonium and triethyl-oxonium salts. Exemplary salt counterions are hexachloro-

An alkylating agent solvent that does not dissolve nylon or render nylon tacky during the alkylation procedure is preferably employed in activating the preferred nylon solid support useful in the diagnostic methods and kits of the present invention. Non-nucleophilic organic solvents, such as dichloromethane, dimethylsulfoxide, tetrahydrofuran, and others, are exemplary solvents that may be employed for this purpose. N-methyl-pyrrolidone is preferred, because it is a solvent that supports alkylation.

The resulting bead surface imidate esters are then reacted under suitable conditions with an amine-containing polymer, whereby amidine residues are formed. Any primary or secondary amine-containing polymer can be employed to form amidine residues, thus covalently immobilizing the polymer onto the surface of the bead. Poly(ethylene-imine), polyallylamine, and polyvinylamine are preferred examples. The preferred solvent used to dissolve the polymer during the conjugation of the polymer to the activated nylon bead is N-methyl-pyrrolidone.

Alternatively, nylon can also be partially hydrolyzed to yield reactive amine or carboxyl groups (capable of subsequently reacting with amine- or carboxyl-containing polymers). In this manner, an activated solid support coated with reactive moieties may be produced.

Dipsticks are preferably employed in the methods and kits of the present invention. Dipsticks having utility in nucleic acid hybridizations indicating G. vaginalis, and including a nonporous bead support and a means for attaching a bead thereto, are so employed. Nonporous bead supports are known in the art. An example of bead attachment to a nonporous bead support involves a perforation or perforations (or a depression or depressions) in the nonporous bead support, wherein beads

can be attached. Preferably, perforations are employed and the beads are attached by pressure fit within the circumference of the such perforations. One of ordinary skill in the art will appreciate that other bead attachment methods may be employed in production of a dipstick useful in practicing the present invention.

The dipstick used in the methods and kits of the present invention may contain more than one bead. Preferably the dipstick will contain from about two to one hundred beads, and more preferably about two to ten beads, each within its own perforation. More preferably, the plurality of bead-containing perforations will be situated in a row along one edge of the dipstick. Such a dipstick can function as an indicator card. Specifically, multiple beads with covalently attached capture oligonucleotide probes or antigens with different sequences or specificities are closely aligned on a multisite dipstick, thereby facilitating the detection of a multiplicity of organisms in a single biological sample. A particular bead may contain oligonucleotides representing a plurality of nonidentical nucleic acid sequences (for example, sequences from a group of related organisms), or a bead may only contain a plurality of identical oligonucleotides having a specific nucleic acid sequence.

Preferably, a dipstick useful in the practice of the present invention will include a bead specific for G. vaginalis, a positive control, a negative control and, optionally, a pH indicator bead or strip. Conventional pH indicators can be absorbed onto or covalently attached to a solid support to be incorporated into a solid phase assay format. See, for example, "Indicators," Bishop (ed.), in Belcher et al. (eds.), "International Series of

in the kit as a separate structural unit, however. Of course, the pH of the test sample will be determined prior to combination of the test sample with one or more solutions of the present invention.

In another embodiment, the dipstick may include beads specific for a gram positive bacteria in addition to a bead specific for G. vaginalis. Such a dipstick will indicate whether the sample cell number is greater than or equal to the critical cell number for these two vaginal pathogens, and will provide a more comprehensive diagnostic tool, since vaginitis may also be detected (and causative organisms distinguished) through the practice of this embodiment of the present invention.

In another embodiment, the dipstick may include beads specific for Neisseria gonorrhoea, Chlamydia spp., Mobiluncus spp., Bacteroides spp., Ureaplasma urealyticum, Prevotella bivia, Group B Streptococci and/or Mycoplasma spp., in addition to a bead specific for G. vaginalis, a procedural positive control, and a procedural negative control. Such a dipstick will indicate whether the sample cell number(s) is equal to or exceeds the critical cell number determined for an individual organism associated with cervicitis (Neisseria gonorrhoea, Chlamydia spp.) or potentially pathogenic vaginal infection. Such dipsticks may be packaged with one or more lysis reagents capable of freeing target nucleic acid sequences for hybridization with the capture and signal probe components of the kits of the present invention. Exemplary dipsticks of this embodiment of the present invention include the following: T. vaginalis, Group B Streptococcus and Prevotella bivia for prenatal risk assessment; G. vaginalis, Mycoplasma hominis and Mobiluncus spp. for bacterial vaginosis; Chlamydia trachomatis and Neisseria gonorrhea for sexually transmitted diseases; and the like.

The development of a dipstick-based assay capable of detecting the presence of multiple target nucleic acids requires identification of three key parameters: (1) the lysis or release of target nucleic acid from the sample; (2) the capture and detection of the target nucleic acid; and (3) the reagent components of the assay format. If the reagent components remain constant (i.e., hybridization conditions, wash solutions, detection enzyme, and the like), one skilled in the art can identify and select specific capture and signal oligonucleotides for hybridization with the target nucleic acid. When the conditions for capture and detection of the target nucleic acid are defined, one skilled in the art can determine lysis conditions that allow the release of the target nucleic acid from a biological sample. The lysis conditions may vary for each of the target microorganisms, and a balance of lytic conditions allows the simultaneous detection of the target organisms. If these parameters are identified, the critical cell number for each microorganism can be determined in biological samples. The critical cell number may vary with the method used to acquire the sample, and can be defined as the cell number that leads to symptomatic presentation of a disorder. Symptomatic presentation is particularly relevant for disorders where an asymptomatic presence of the microorganism is possible.

In a further embodiment of the claimed invention, oligonucleotide-coated beads may be employed in a micro-titer well format. This format may be advantageously used when a large number of patient samples are assayed. Further, the microtiter well format is compatible with signal probes that are detected through

obtained when exemplary lysis reagents were incubated with the indicated organisms in the presence of patient samples. The first method, designated (1), involves direct immersion of the sample in 3 M guanidinium thiocyanate (GuSCN). The second method (2) requires heating to 65°C in 1 mg/ml proteinase K, followed by the addition of GuSCN to a final concentration of 3 M. The third method (3) requires heating at 85°C in a buffered solution containing detergents, followed by addition of GuSCN to a final concentration of 3 M.

TABLE 1

LYSIS REAGENTS

Organism	(1)	(2)	(3)
Candida	-	+	+
Candida, spiked[a]	-	+	+
Gardnerella	-/+	+	+
Gardnerella, spiked[a]	-	+	+
Trichomonas	+	+	+
Trichomonas, spiked[a]	+	-	+

[a] "Spiked" means a swab was taken from a BV-negative woman, and about 5 x 10^7 cultured organisms were placed directly onto the swab. The swab is then processed as if it were a patient sample.

A plus sign indicates successful detection of target nucleic acid in the presence of vaginal fluid samples. Using these reagents, patient samples are collected into proteinase K followed by GuSCN addition (method (2)) for Candida albicans and G. vaginalis detection, and into 3 M GuSCN (method (1)) for Trichomonas vaginalis detection. Alternatively, two patient samples are collected simultaneously, or one patient sample would be divided into aliquots, and incubated in 5 M GuSCN and 1 mg/ml proteinase K, respectively. The two solutions are then mixed to a final GuSCN concentration of 3 M. The sandwich assay is then performed on this mixture. Still another alternative involves the identification and use of a lysis reagent useful for the release of nucleic acid from all organisms of interest (method 3, described above) The components of the buffered detergent solution, heating temperature, and time of heating may be adjusted for each organism individually or for the organisms in combination.

primarily in terms of nucleic acid hybridization assays, many other uses for these dipsticks are contemplated. Any member of a ligand pair can be attached to beads in the dipstick, and the dipstick can then be used to identify the corresponding ligand member. For example, antigens or antibodies could be attached to beads, as described above, in a dipstick, and then corresponding antibodies or antigens, respectively, could be identified. In a similar manner, other ligand systems, such as biotin and streptavidin, can be used.

The diagnostic methods of the present invention correlate well with traditional clinical diagnoses of BV. Illustrative patient samples used for comparative diagnostic testing were obtained from single, white females in their 20's at the Student Health Clinic at the University of Washington. The "normal" (i.e., BV-negative) patients were attending the clinic for routine examinations and showed no symptoms of vaginitis. pH was routinely measured at the time the samples were taken.

In these studies, a vaginal sample was obtained and analyzed in accordance with conventional techniques to obtain a conventional BV diagnosis. Subsequently, a vaginal wash was obtained and a portion of this sample was mixed with concentrated 5 M GuSCN solution to yield a final concentration of 3 M GuSCN. The sample was concentrated onto nitrocellulose paper by slot blot techniques and then hybridized with a radiolabeled, G. vaginalis-oligonucleotide probe (i.e., the probe hybridizes specifically with 16S rRNA of G. vaginalis when challenged with more than 70 other potentially cross-reacting species that may be found in the normal microflora of the vagina). Hybridization of ^{32}P-labeled probe to each sample was determined by autoradiography, and levels of probe hybridization to samples and standards

were compared.

A comparison of patient sample slot blot results with standards of known target nucleic acid concentrations allowed quantitative determination of the number of G. vaginalis cells in the patient samples. When the pH (greater than 4.5) and number of G. vaginalis cells (greater than or equal to approximately 2 x 10^7) diagnostic criteria of the present invention were used in evaluating the slot blots, BV was detected with a sensitivity of 95.3% and a specificity of 98.6.%. These diagnostic comparative results are set forth in Example 5 below.

The preferred diagnostic methods and kits of the present invention function through hybridization reactions, which constitute the established method for identifying specific nucleic acid sequences. Hybridization is based upon the pairing of complementary nucleic acid strands. When complementary single stranded nucleic acids are incubated in appropriate buffer solutions and conditions, complementary nucleotide sequences pair to form stable, double stranded molecules (i.e., the sequences hybridize).

The particular hybridization technique employed is not essential to the methods and kits of the present invention, and one of ordinary skill in the art will appreciate the variety of such techniques. Hybridization techniques are generally described in Hames et al. (eds.), "Nucleic Acid Hybridization, A Practical Approach", IRL Press, New York, 1985. As improvements are made in hybridization techniques, they will be readily applicable to the present invention. In addition, diagnostic dipstick and immunoassay technologies are known and employed in the art for a variety of purposes.

second hybridization with a signal oligonucleotide probe must occur. In this manner, the presence of captured target nucleic acid is confirmed, and the amount of captured target nucleic acid may be quantified. The detection of successful or unsuccessful hybridizations may be accomplished in accordance with methods known to practitioners in the art, such as colorimetric, chemiluminescent or fluorescent means.

Indirect quenching fluoroimmunoassay, double receptor fluoroimmunoassay, or protection fluoroimmunoassay are known assay formats that use antibodies directed against the fluorescer. These assays are based on competition for the fluorescer-labeled antigen by the antigen-specific antibody and the fluorescer-specific antibody.

As described in copending U.S. Patent Applications Serial Nos. 142,106, 444,872, 522,442, and 571,563, oligonucleotides covalently immobilized on polymer-coated beads or similar structures can serve as nucleic acid probes. Hybridization assays that detect the presence of specific target nucleic acid in complex biological samples can be conducted utilizing such immobilized oligonucleotide probes.

For the purposes of this description, the term "oligonucleotide" refers to a short nucleic acid sequence that is approximately 6 to 150 bases in length. Such oligonucleotides can be used as capture or signal probes in hybridization assays, and are preferably chemically synthesized using commercially available methods and equipment. For example, the solid phase phosphoramidite method can be used to produce short probes of between 6 and 100 bases having a molecular weight of less than 16,000 daltons. For the synthesis of oligonucleotides, see generally Caruthers et al., Cold Spring Harbour Symp.

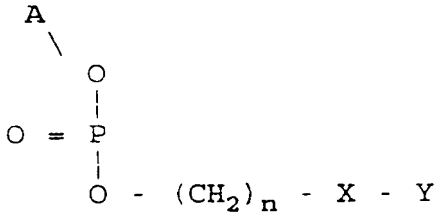
Quant. Biol. 47:411-18, 1982; and Adams et al., J. Am. Chem. Soc. 105:661, 1983. Activated oligonucleotides refer in general to oligonucleotides that have been reacted with a chemical compound and rendered chemically active. For the purposes of this description, the term "activatable" refers to the potential of a moiety to become chemically reactive.

When synthesizing an oligonucleotide probe for detection of a specific target nucleic acid, such as G. vaginalis RNA, DNA or the like, the choice of nucleotide sequence will determine the specificity of tests using such probes. For example, by comparing nucleic acid sequences from G. vaginalis isolates, one can select an oligonucleotide sequence for G. vaginalis detection that is either type-specific, species-specific or genus-specific. Comparisons of nucleic acid regions and sequences can be conducted using commercially available computer programs.

Oligonucleotide probes useful in the present invention are capable of hybridizing with a nucleic acid sequence specific for G. vaginalis; 16S G. vaginalis rRNA is a preferred G. vaginalis nucleic acid target for this purpose, because it is present in several thousand copies per cell. However, oligonucleotides complementary to sequences in the G. vaginalis genome or in G. vaginalis plasmids may also be employed. In addition, oligonucleotide probes capable of hybridizing with nucleic acid sequences specific for Candida spp., Group B Streptococci, Prevotella bivia, Ureaplasma urealyticum, Mobiluncus spp., Mycoplasma spp., Neisseria gonorrhea, Chlamydia spp. and Trichomonas vaginalis are described. Exemplary oligonucleotide probes useful in the present

The preferred capture oligonucleotides for use in the present invention are synthetic oligonucleotides from about 15 to about 100 bases in length. A spacer (linker) arm (i.e., a chemical moiety that extends or links other chemical groups, and preferably is a carbon chain containing from about 2 to about 12 carbon atoms, more preferably about 6 carbon atoms) containing a blocked amine group can be coupled, during oligonucleotide synthesis using conventional chemistry, to the 5'-hydroxyl group of an oligonucleotide. A primary amine is the preferred group for reaction with monofunctional or multifunctional reagents, and its attachment via a hexyl arm is preferred. Reagents useful for the attachment of spacer arms terminating in a primary amine are commercially available. Starting materials suitable for use in accordance with the present invention are known in the art.

Preferably, an oligonucleotide possessing a 5'-terminal structure, such as



is employed, wherein n is 1-12, inclusive (n = 6 preferred); X is -NH- or -NHC:O$(CH_2)_m$NH-, wherein m is 2-12, inclusive; Y is 4,6-dichlorotriazine (preferred) or a thiol (sulfhydryl)-reactive moiety; A is an oligonucleotide, ranging from about 9 to about 100 bases, preferably from about 15 to about 30 bases, with only the 5'-hydroxyl oligonucleotide moiety requiring modification for attachment. Alternatively, the 3' end of the oligonucleotide may be similarly modified to contain a reactive

group.

Selected oligonucleotides are then activated with a monofunctional or multifunctional reagent. Exemplary reagents include homotrifunctional, heterotrifunctional, homobifunctional, and heterobifunctional reagents. Selected oligonucleotides may be activated and linked to polymer-coated solid supports according to the following chemistries. An amine-tailed oligonucleotide can be activated with a monofunctional or multifunctional reagent (e.g., cyanuric chloride). The alkylamino dichloro-triazine formed in the activation step is reactive toward the amine-containing polymer bound to the solid support.

Although cyanuric chloride, a homotrifunctional reagent, is preferred, other activating reagents can be used. For example, N-succinimidyl-4-(iodoacetamido)-benzoate (SIAB) is a suitable heterobifunctional reagent, and disuccinimidyl suberate is a suitable homobifunctional reagent. If solid support carboxyl groups are involved, the heterobifunctional reagent 1-ethyl-3-(dimethyl-aminopropyl)-carbodiimide can be used. Other similar monofunctional and multifunctional (heteromultifunctional and homomultifunctional) reagents are suitable for use in the practice of the present invention.

The oligonucleotide activation and linking chemistries result in the selective activation of an amino group on an oligonucleotide, without modification of any of the purine and pyrimidine bases of the oligonucleotide. Specifically, the preferred chemistry employs cyanuric chloride (2,4,6,-trichloro-1,3,5,-triazine). Oligonucleotides possessing a 5' or 3' tethered (via a hexyl arm) nucleophilic amine moiety (or internal aminoalkyl groups substituted on pyrimidine or purine

preferably, 19-25°C in a part organic solvent, such as N-methyl pyrrolidone, for 1 to 2 hours.

The unreacted cyanuric chloride can be removed by exclusion chromatography or ultrafiltration. The treated solid support and activated oligonucleotide are then conjugated. Specifically, they are mixed together and preferably incubated at from about 20 to 50°C for about 1 to 24 hours. The residual (unreacted) amines on the bead surface can be capped (blocked) with an agent, such as succinic anhydride, preferably in N-methyl pyrrolidone in the presence of an appropriate base, such as sodium borate, to render the surface compatible (negatively charged) for nucleic acid hybridization. It should be noted that the solid support may be chemically modified to produce a positive, negative, or neutral surface charge.

The target nucleic acid is usually a polynucleotide with an average length ranging from about 20 to about 20,000 bases or nucleotides in length. Suitable conditions for hybridization relate to stringency conditions wherein base-pairing mismatching is minimized or non-existent. The degree of acceptable mismatching is dependent upon the specificity required, in a manner recognized by a practitioner in the art.

Signal oligonucleotide probes useful in accordance with the preferred methods and kits of the present invention are oligonucleotides conjugated to or conjugable with detectable labels. Various labels can be used in hybridization assays of this invention. Such labels act as reporter groups for detecting duplex formation between the target nucleic acid and its complementary signal sequence. A reporter group as used herein is a group having a physical or chemical characteristic that can be measured or detected. Detectability may be provided by such characteristics as enzymatic activity, color change,

luminescence, fluorescence, or radioactivity, or it may be provided by the ability of the reporter group to serve as a ligand recognition site. Any haptenic or antigenic compound can be used in combination with a suitably labeled antibody for this purpose.

Exemplary enzymes of interest as reporter groups are hydrolases, particularly phosphatases, esterases, ureases, and glycosidases, oxidoreductases, particularly peroxidases, and the like. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, and the like. Chemiluminescers include luciferin, luminol, oxetane-diones, and the like. The above list is illustrative only, and the choice of label depends on sensitivity requirements, ease of conjugation with the probe, stability requirements, and available instrumentation.

The extent of hybridization may be quantified using a method or technique of fluorescent quenching, described in copending U.S. Patent Application Serial No. 558,967. Dipstick (i.e., insoluble) diagnostic format fluorescent quenching employs a solid support, such as nylon, that fluoresces when irradiated with ultraviolet light (240 to 400 nanometers (nm)). Colorimetric insoluble enzymatic product deposited on the solid support during the preferred assay procedure quenches the fluorescence of the solid support, thereby providing a means of quantifying the amount of product using commercially available fluorometers. The solid supports described herein do not require a plurality of fluorescent chromophoric groups to be bound thereto, but rather rely on the intrinsic or natural fluorescence of the polymeric material forming the solid support. The irradiation and detection of

the use of narrow or specific wavelengths of ultraviolet or visible light.

For example, a sandwich may be formed in which a target nucleic acid is hybridized to the solid support; a signal biotinylated-oligonucleotide is then hybridized to the target nucleic acid; and reporter enzyme conjugated to streptavidin is bound to the biotinylated oligonucleotide. After sandwich formation, reporter enzyme product is allowed to deposit or accumulate on the surface of the solid support. In most cases, the quantity of enzymatic product produced is directly proportional to the quantity of captured target nucleic acid. The solid support is then irradiated with an ultraviolet light source (240 to 400 nm) and the resultant fluorescence is determined with a fluorometer. The intensity of the measured fluorescence is inversely proportional to the quantity of enzymatic product deposited on the solid support. Alternatively, if the reporter enzyme product is colored, product deposited or accumulated on the surface of the bead can be qualitatively and/or quantitatively determined. Quantitative determinations may be performed visually or by an instrument capable of analyzing and/or measuring gradations of color, either directly or using shades of grey. Such quantitative determinations generally include a comparison to standards.

If the fluorescence quenching method is used, the only required property of an enzymatic product useful in quantifying the amount of captured target nucleic acid is the ability to quench or mask the fluorescence of the solid support. Any type of enzyme which produces a colorimetric product can be utilized in the fluorescent quenching assay. Exemplary enzymes include horseradish peroxidase (HRP), alkaline phosphatase (AP), beta-galactosidase and the like. Representative substrates for

each exemplary enzyme include 4-methoxynaphthol (4MN), 5-bromo-4-chloroindoyl-3-phosphate/nitroBlue tetrasolium (NBT), and o-nitrophenyl-beta-D-galactopyranoside (ONG), respectively.

Since the quantity of enzymatic product produced is proportional to the quantity of captured target nucleic acid, and the quenching of fluorescence by the colored product is proportional to the quantity of product produced, a quantitative determination of captured target nucleic acid can be made. In most cases, the relation between captured target nucleic acid and fluorescence quenching is linear.

In this embodiment, the solid support must necessarily possess some type of fluorescence when irradiated by ultraviolet or visible light. The fluorescence can be intrinsic or inherent to the material composing the solid support, or fluorescent compounds can be bound (either covalently or non-covalently) to the solid support during the manufacturing or derivitization process. Exemplary fluorescent compounds include fluorescein, Texas Red, rhodamine and the like.

Dipstick format diagnostics of the present invention may be evaluated by visual assessment only. In this embodiment, the bead will turn from its natural color to an indicator color, such as blue, to indicate positive results. For example, a dipstick might be designed to turn an indicator color visible to the eye when a sufficient amount of a target nucleic acid is present. This embodiment of the present invention will not require irradiation of the sample with ultraviolet light or detection with a fluorometer.

Alternatively, direct determination or quantitation

present invention. To obtain accurate results, however, the fluorescent enzymatic product must be measured in an environment that neither quenches the product nor is fluorescent itself. Since the preferred solid supports described herein possess a very high intrinsic fluorescence in their native state, it is not possible to accurately measure a fluorescent soluble enzymatic product in the presence of the preferred solid support. Such a solid support must therefore be removed from the substrate solution or the solution decanted and placed in a separate vessel to allow accurate determination of fluorescence.

Alternatively, for fluorescent signal detection, colored or coated polymeric beads may be employed. Coloring the preferred solid support with a dye of any color significantly reduces or quenches the intrinsic fluorescence thereof. Coating serves to mask any intrinsic fluorescence of a particular solid support. This reduction in fluorescence permits the solid support to be present during soluble enzymatic product fluorescence measurement, thereby obviating the need to transfer solutions or solid supports. Fluorescent signal detection may also be accomplished by employing microtiter wells directly coupled to the capture nucleic acid sequence.

If a bead or solid support (preferably dyed or colored) remains in solution during fluorescent signal detection, bead-to-bead or solid support-to-solid support fluorescence must be relatively uniform. The standard deviation of bead-to-bead intrinsic fluorescence should not exceed the standard deviation of the assay (typically 1 to 10%) or of the detected fluorescence (typically 1 to 5%), in order to fully utilize the potential of the fluorescent substrate. This procedure is used in the microtiter well format of the present invention, where a

soluble fluorescent product is measured directly in the presence of the colored bead. Less meaningful measurements are obtained when deviations greater than those set forth above occur.

Virtually any color of dichlorotriazine, azo, or other permanent dyes will reduce the intrinsic fluorescence of the nylon approximately 500-fold. Exemplary dye colors are black, blue, red, green, yellow, purple, orange and the like, with the only requirement with respect to dye color being that the dye not fluoresce at the same wavelength as the fluorescent enzymatic product.

In addition to quenching the natural fluorescence of the preferred solid support, coloring the supports allows the development of solid supports with different capture oligonucleotides or different target nucleic acid specificities that are distinguishable by color. The ability to distinguish different solid supports by color has the following advantages: 1) quality control can be enhanced, because solid supports possessing different specificities can be identified and distinguished; and 2) contrast between a colorimetric enzymatic product and the surface of the solid support can be maximized. This allows a greater level of sensitivity to be achieved when assay results are determined by visual inspection.

The kits of the present invention can be used as part of a semi-automated method for BV diagnosis in accordance with the present invention. Such kits would provide for the detection of G. vaginalis when present at or above the level characteristic of BV, and may include a sample obtaining means, such as a vaginal swab; a pH indicating means, such as pH paper; lysis

such as a diagnostic dipstick. This kit may be used in conjunction with an incubation apparatus (such as a heating block), an automated assay device and/or a bead reader.

A practitioner using the semi-automated method of the present invention for the diagnosis of BV will follow a procedure substantially as described below:

1) Obtain a vaginal swab and apply the swab to provided pH paper.

2) Insert the swab into a tube containing lysis solution and soak for approximately five minutes or less.

3) Squeeze out the swab, and optionally place the tube into a well of a heating block, heating at approximately 65°C for approximately five to twenty minutes.

4) Add 5 M GuSCN solution to a final concentration of 3 M.

5) Transfer the solution to the first sample well in an automated dipstick processor, which automatically completes the assay development in approximately 30 minutes.

6) Visually determine whether a colored substance has been deposited on the bead.

In this semi-automated methodology, step 1 corresponds to determining pH of a vaginal sample obtained from the patient. Step 2 may use a proteinase K lysis buffer. Steps 2-6 constitute determining whether the critical G. vaginalis cell number is equalled or exceeded by the patient sample.

These methods and kits are advantageous in that they provide accurate BV diagnosis rapidly and reproducibly, without a requirement for highly skilled, labor-intensive analysis. The methods of the present invention may be accomplished in 6 hours or less, and preferably are

accomplished in 1 hour or less.

Also, the methods and kits of the present invention eliminate the need for skill in identifying clue cells or evaluating wet mounts. Consequently, the methods of the present invention could be practiced and the kits of the present invention could be used by individuals without the aforementioned skills. Specifically, a laboratory technician or physician's assistant could employ the methods and kits of the present invention with virtually no special training. Another ramification of using the methods and kits of the present invention is that the subjectivity involved in wet mount and Gram stain analysis is replaced with more objective procedures. Specifically, analyst-to-analyst variation is eliminated through the use of the present invention.

In the physician's office setting, physical examinations and wet mounts are primarily relied upon to diagnose BV. These methodologies are less accurate than the gold standard method, but the gold standard method is generally considered too complicated to perform in an office environment. The methods and kits of the present invention can be utilized in the office setting to achieve diagnostic results comparable to those provided by the gold standard method or the physical examination/wet mount method.

In an alternative embodiment, patient samples may be collected, processed and analyzed to simultaneously detect the presence of Gardnerella vaginalis, Candida spp., and/or Trichomonas vaginalis. Samples are collected from symptomatic females presenting with vaginal complaint who have not been treated with anti-bacterial or anti-fungal medication within the week prior to sample collection and

obtain vaginal fluid samples. Dacron swabs with pre-scored handles are particularly preferred for sample collection. Samples are obtained by twisting or rolling the swab against the vaginal wall two or three times, ensuring that the entire circumference of the swab has touched the vaginal wall. The swab is then placed in a sample collection tube, the pre-scored handle of the swab is broken, and the tube is capped. The unlubricated speculum is removed from the patient, and the vaginal pH is determined by touching a pH indicator strip to the speculum.

In a protocol for immediate sample preparation, the swab/sample is transported immediately at room temperature for processing and analysis. If the swab/ sample cannot be immediately processed, the swab/sample is held at 0°C to 8°C for four hours or at room temperature for one hour.

For processing, lysis solution is added to the swab/sample, which is then swirled or agitated in the lysis solution for about 10-15 seconds. The tube containing the swab and lysis solution is heated at 85°C for 5 minutes, and hybridization solution is mixed with the sample. At this point, samples may be stored for up to 24 hours at room temperature.

The swab contents are expressed by twirling the swab against the side of the tube, and the solution remaining in the tube may then be processed on an automated instrument. Generally, the solution remaining in the tube is filtered prior to further processing. In a preferred embodiment, the sample is placed in the first well of a reagent cassette or multi-cavity container, and the semi-automated instrument moves a dipstick through each well of the cassette, thereby processing the sample. A preferred dipstick contains five beads – a procedural control, a negative control, a bead with Gardnerella vaginalis-

specific capture probe, a bead with Candida spp.-specific capture probe, and/or a bead with Trichomonas vaginalis-specific capture probe. It is preferred that the detection of these three organisms be achieved through a colorimetric signal system, wherein the presence of color on a test bead at the end of automated sample processing is indicative of a detectable level of nucleic acid from that target organism. The intensity of color on a bead can be estimated either visually or by a measuring device, such as an image analyzer or reflectometer. The procedural control ensures that the procedure has been correctly performed and serves as a reagent quality check. The negative control evaluates non-specific binding to the beads.

In the experimental section below, Example 1 describes quantitation of horseradish peroxidase insoluble product on 3/32nd inch nylon beads using the fluorescence quenching technique. Example 2 describes the quantitation of alkaline phosphatase insoluble product on 3/32nd inch nylon beads using the fluorescence quenching technique. Example 3 describes a comparison of direct fluorescence using soluble 4-methyl-umbelliferone in the presence of black and natural colored nylon beads. Example 4 describes the reduction in fluorescence of nylons beads by dying the beads with a multiplicity of different colors. Example 5 illustrates the correlation between the diagnostic criteria employed in the practice of the present invention and conventional BV diagnostic techniques. Example 6 describes methods and kits for simultaneous detection of Gardnerella vaginalis, Candida spp., and Trichomonas vaginalis target nucleic acid in a complex biological sample. Example 7 describes improved

nucleic acids in a complex biological sample.

The following Materials and Procedures sections pertain to the above-summarized Examples 1-5.

MATERIALS

Solutions:

APB buffer is 0.18 M NaCl, 0.05 M Tris-HCl, pH 7.6, 5 mM EDTA, and 0.5% (v/v) Tween® 20.

TMNZ buffer is 0.05 M Tris, pH 9.5, 1 mM $MgCl_2$, and 0.5 mM $ZnCl_2$

FW (filter wash) is 0.09 M sodium chloride, 50 mM Tris, pH 7.6, and 25 mM EDTA.

SDS/FW is FW and 0.1% (w/v) sodium dodecyl sulfate (SDS).

HRP (horseradish peroxidase) substrate solution is 0.1 M sodium citrate, pH 6.5, 0.2 M sodium phosphate, 0.5 mg/ml 4-methoxy-1-naphthol, 0.02 mg/ml 3-methyl-2-benzothiazolinone hydrazone and 0.0135% (v/v) hydrogen peroxide.

AP (alkaline phosphatase) substrate solution is 1 mM 5-bromo-4-chloroindoyl-3-phosphate, 1 mM nitroBlue tetrazolium, and 0.01% (v/v) Tween® 20 in TMNZ.

5 M GuSCN is 5 M guanidinium thiocyanate, 83.5 mM Tris, pH 8.0, 8.35% formamide, and 16.7 mM EDTA.

3 M GuSCN is 3 M guanidinium thiocyanate, 50 mM Tris, pH 8.0, 5% formamide, and 10 mM EDTA.

Proteinase K lysis solution is 1 mg/ml proteinase K, 0.5% (w/v) SDS, and 5% N-lauroylsarcosine (sarcosyl).

Lysis and hybridization solution is 3 M guanidinium thiocyanate (GuSCN), 50 mM Tris, pH 7.6, 2% sarcosyl, and 25 mM EDTA.

Hybridization/slot blot solution is 90 mM Tris, pH 8.0, 0.6 M NaCl. 10 mM EDTA, 0.5% SDS, 5X Denhardt's (1X